Antimicrobial Potential of Biosurfactant Isolated From Oil-Degrading Bacteria Against Multi-Drug-Resistant Pathogens

Anita Shafiee Sabet, Farzanegan 1 High School,Tehran/Iran , anita.shafiee.79@gmail.com

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ABSTRACT

Hospital acquired infections are still a serious and common issue around the world leading to many undesirable consequences and even death of considerable percent of patients. Among the possible solutions, using biosurfactants seem to result in promising outcomes. The biosurfactant used in present work is derived out of existing bacteria in oil-contaminated soils in different refinery sites in Iran. The results of the antimicrobial activities of biosurfactants against Staphylococcus aureus and Pseudomonas aeruginosa were promising. Therefore, isolated strain of oil-contaminated soil may be a valuable candidate of favorable biosurfactant-producing bacteria for the inhabitation of infectious bacteria.

1 Introduction

Nowadays, hospital acquired infections are the origin of some usual diseases. These diseases could result in extending the healing process or even death depending on the microbe and the defense system of the patient. Usually the weaker people like babies, old people and cancer patients are more affected by these infections in hospitals [1]. Currently, there are some methods in order to overcome these happenings such as recognizing these infections and disinfecting using Ultra Violet (UV) light etc. [2] One type of the hospital acquired infections happens because of the bacterial biofilm. Biofilm is a stable bacterial colony with the possibility of cytoplasmic collaboration on a surface that is surrounded by a matrix which is made of polymer material outside the cell having microbial base. In this study, biosurfactants are used in order to overcome these issues. Surface active agents (biosurfactants) are materials that could diminish the existing surface tensions between surfaces because of their bipolar molecular structure [3]. The biosurfactant compounds are widely used in pharmaceutical industries. Besides, these materials are biodegradable and have less toxicity comparing with synthesized surfactants. In this study, the biosurfactants are derived out from oil contaminated soils as a bacterial source and purified. The oil contaminated soils were collected from various locations of Iran and the influence of produced biosurfactant was investigated.

2 Literature review

There have been done a lot of researches during past years to control the common existing bacterial biofilms in hospital environments such as Staphylococcus Aureus and Pseudomonas Aeruginosa. According to Rienzo *et al.* (2016), the antibacterial effects of biosurfactant on gram-positive bacteria (Staphylococcus Aureus) was more than Pseudomonas Aeruginosa's biofilm [4]. Primo *et al.* (2015), showed that the effects of biosurfactant were very remarkable on movement, signaling and structure of herbal biofilms [5]. Ciandrini *et al.* (2016), figured out that decomposed biosurfactant could reduce the surface tension of oil paraffin [6]. Mostafapour *et al.* (2014), implemented researches in order to identify a bacterial strain of Bacillus Cereus that produces biosurfactant and evaluated its antibacterial characteristics. In this study different samples were considered such as water, oil and oil containing soil. The ability to make emulsion and surface tension properties were considered using biochemistry tests. According to their results Bacillus Cereus 43 showed a good reduction in surface tension and the derived biosurfactant from this sample had desirable antibacterial effects. Thus they have the potential to be used in biotechnology and bioenvironmental applications [7].
Shahaliyan *et al.* (2013), analyzed the sediments to compare the biosurfactant growth and production of living bacteria in some oil contaminated sediments. The results show that the Pseudomonas growth as a biosurfactant producing bacteria was more than Alcaligenes denitrificans. This was because of increasing the solubility of anthracene and making this hydrocarbon available [8].
Safari *et al.* (2011), worked on deriving the microorganisms that could produce biosurfactant and their properties on the surface. One of the ten samples in this study had the possibility of biosurfactant production in big amounts. This bacteria was named Caspian Petroleum A1 (CPA1). This strain (CPA1) was defined as a biosurfactant producing bacteria having the ability of decomposing gas oil, oil, anthracene and naphthalene [9].

3 Experimental Methods

In this study, 6 aerobe bacteria were derived from oil contaminated soils of different parts of Iran and were purified during several steps. The produced biosurfactant were tested in oil spreading tests. Three samples showed desirable results. Then the tests were done in order to understand their effects and properties.

3-1 Sampling

Due to the properties of different biosurfactants in microorganisms it seems possible to reach the microorganisms which could produce these compounds from different regions. In fact, there is a higher chance to isolate microorganisms producing biosurfactant from the soils containing hydrophobic substances. Accordingly, the separation took place on oil contaminated soils of regions like Qom, Shazand Arak refinery, Khark, Gachsaran, Siri

and Khangiran. Then, the soils were grounded using a mortar, and big particles of the samples were removed. In order to protect the existing microbes in the soils, they were remained in room temperature (25°C).

In this study, enrichment is used in order to separate the bacteria which produce biosurfactant from the soils contaminated by hydrophobic compounds.

3-2 Microorganism separation using enrichment method with hydrocarbon substrate

This method is based on creating a proper condition for growth of the desired isolate and making the conditions bad for the other undesirable bacteria. In order to perform the enrichment, the bushnellhas broth containing 1% crude oil as a carbon source was used. The homogenized soil samples, a part of Bushnellhas broth and 1 % crude oil were poured in Erlenmeyer flask and kept in shaker for one week in 25 °C and 180 rpm. Next, 100 µl of each Erlenmeyer flask added to nutrient agar and cultured and the plates incubated at 25 °C for one week, and each day the plates were monitored in order to find new colonies [6].

3-3 Purification of the microbial isolates

After proper heating the nutrient agar plates, the grown microbial isolates were purified individually and the repetitive isolates were identified.

3-4 Biosurfactant production using selected microbes in liquid culture

In this test to produce the biosurfactant, a little part of each culture medium were poured in different plates and kept for 7 days at 29 °C. A plate of new microbial culture added to each plate. Heating implemented for 7 days in shaker incubator at 25 °C and 180 rpm. Then, in order to separate the biomass from the fermentation liquid each plate was centrifuged with 4000 rpm, and the upper liquid was transmitted through filter paper number 1. The remained existing oil on upper liquid layer was separated using decanter. Finally, existence of the biosurfactants in each sample was evaluated using oil and parafilm spreading tests.

3-5 Investigation of produced biosurfactant using oil spreading test

This method is a useful and rapid way to early evaluation of the strain that have the ability to produce biosurfactant. In this process, 40 µl distilled water was poured in a glass plate at first. Then, 50 µl of crude oil was placed on the center of the plate in order to create a layer of oil on the existing water. Next, 30 µl of upper layer of the centrifuged liquid was poured on the center of the oil layer. This way, the movement of oil from the center to outer area was considered as a criterion of biosurfactant production. The resulting halo diameter in this method depends on the biosurfactant viscosity and this method could be considered as a qualitative test to evaluate the produced biosurfactant [10].

3-6 parafilm

In this method 25 µl of upper layer of the produced liquid from centrifuge was settled on hydrophobic parafilm. The resultant droplet should be dispersed if there is biosurfactant. But if there is no surface active compounds the droplet remains with its early geometry and will not be dispersed. This could also be used as a criterion of existence of the biosurfactant.

3-7 Biosurfactant extraction

In order to extract the biosurfactant, the colony of the selected isolate was added to a 1000 ml Erlenmeyer . Then, the mixed liquid was kept in 25 °C of heating and 150 rpm for 7 days. The bacterial cell were removed after 20 min of 4000 rpm centrifuge in 5 °C and the upper layer was stored. The pH of liquid was set to 2:1 M by using sulfuric acid. Afterwards, chloroform and methanol was added having the similar volume fraction (1:2). The organic phase was separated and was vapored in 60 °C in oven. The final product was brown as crude biosurfactant [3].

3-8 Antibacterial evaluation of the biosurfactant

The produced suspension of biosurfactant was used to evaluate the antibacterial effect. Spreading in agar was utilized as a qualitative method. In this method, the standardized microbial suspension was spread on Mueller-Hinton agar. Then, for antibacterial analysis, paper discs, having appropriate distance from each other, were put on the plate and about 20 µl of solvent were poured on the discs. Next, the culture medium containing the bacteria remained for 24 h in 37 °C in the incubator. Eventually, measuring the resulting halo diameters around the discs was performed [6].

4 Materials and Methods

4-1 Sampling

In this step 10 soil samples were gathered from Qom, Shazand Arak refinery, Khark, Maroon, Gachsaran, Siri and a gas contaminated soil sample from Khangiran, and the samples were delivered to the laboratory in shortest time.

4-2 Preparation of liquid culture environment

300 ml Bushnellhas broth was prepared containing mineral substances, oil as carbon source and peptone as nitrogen source for bacteria nutrition. Then poured in six 50 ml Erlenmeyer flask . Then, 0.5 g of the samples was added in each Erlenmeyer and they were remained for one week in shaker in order to prevent the sediment formation and better air feeding to the aerobic bacteria (Fig. 1).

Fig. 1: The liquid culture medium before sterilization, the samples on a shaker, adding soil to culture medium; three different procedures (from left to right)

4-3 Screening of bacteria

In this step, 50 ml of the samples was poured on nutrient agar. The plates were remained in incubator in 37 °C for one week and were observed each 24 h to be informed about the new colonies before their unlimited growth.

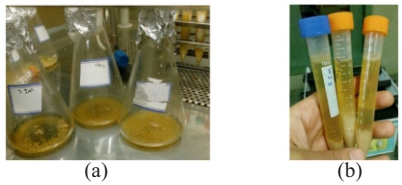
4-4 Purification of the colonies from the culture medium

The colonies were purified by mean of four step culturing using loop in nutrient agar (Fig.2). At first, their shape and color were used for distinguishing. Then, they were compared by Gram staining.

Fig.2: Purified colonies

4-5 Biosurfactant production test

Biosurfactant production tests were needed in order to analyze the productivity of each sample's biosurfactant product. For this purpose, a culture medium was prepared containing peptone, as Nitrogen source, and olive oil in little amount, as carbon source (Fig.3 a/b). The carbon source in this step was less than the previous step because here it was used to help initial growth of bacteria while in the previous step it was utilized to make the growth of strong bacteria as hard as possible.



(a) (b)

Fig.3: a) The produced biosurfactant , b) after centrifuge

4-6 Biosurfactant effectiveness test

After seven days, the samples were centrifuged to create sediment of bacteria on the bottom and be separated from the produced biosurfactant. For effectiveness tests oil spreading and parafilm tests were utilized. For oil spreading test, 40 ml of water was poured in six plates. Then, 50 µl oil and 30 µl biosurfactant sample added to each plate. The resultant halo diameter was measured and considered as an effectiveness criteria of the produced biosurfactant. Figure (4) , and Table 1 demonstrate the results of oil spreading tests for different samples.

As the parafilm test, 10 µl of water was poured on a parafilm layer as the negative control sample and then 10 µl of produced biosurfactant samples put on the parafilm in order to observe the influence of biosurfactant on parafilm.

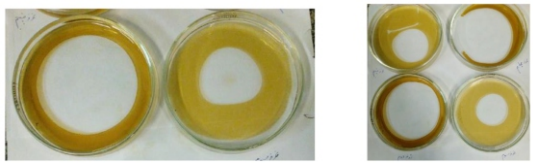


Fig. 4: Comparing the samples by the halo diameter

4-7 Influence of biosurfactant on pathogenic bacteria

Firstly, each of Staphylococcus Aureus and Pseudomonas Aeruginosa were solved in 100 µl of physiologic serum. To be sure about the homogeneity a vortex was used. Spectrophotometer was employed to evaluate the viscosity of the solvent. This amount should be between 1.5 and 2 OD, then each bacteria was cultured on solid nutrient agar. In order to evaluate the influences on bacteria the disc method was utilized.

A few minutes was spent for settling down of bacteria on the environment. For each plate, disc of containing the sample, disc containing antibiotic and one disc containing the solvent as the control sample was used. The solvent was 3 h in refrigerator to spread in the environment and then they were put in the incubator of 37 °C. After one day, result of the zone of inhibition showed 15 mm of diameter (Fig. 5).

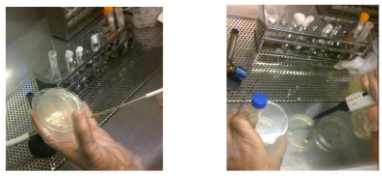


Fig. 5: Subculturing the infectious bacteria and Well test; from left to right

5 Results and discussions

5.1. Oil spreading test

The oil spreading test results is shown in Table (1).

Table 1: Oil spreading test results

Halo diameter (cm)	Sample
10	Ash(1)
5	A1S6
1.5	Si
7	Asha(3)
7.5	Ash(2)
4	Sh(1)
1	Control

5-2 Antibacterial effect of the biosurfactant

The most effective sample was utilized for test on the suspension of separated pathogenic bacteria (Fig. 6). The outcome result showed that the produced biosurfactant out of Ash(1) bacteria had more effects on Pseudomonas Aeruginosa and showed a halo of 1.5 cm.

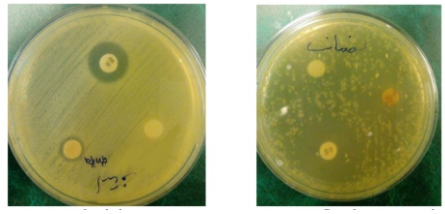


Fig 6: Staphylococcus aureus and Pseudomonas aeruginosa (left to right)

6 Conclusion

According to the results of this project, isolated strain of oil-contaminated soil may be a valuable candidate of favorable biosurfactant-producing bacteria for the inhabitation of infectious and multi-drug-resistant bacterias.

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